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(12) UK Patent Application (19) GB (11) 2 140 822 A

(43) Application published 5 Dec 1984

(21) Application No 8411838

(22) Date of filing 9 May 1984

(30) Priority data
(31) 493835 (32) 12 May 1983 (33) US

(51) INT CL³
C12N 15/00

(52) Domestic classification
C6F GD GF HAX

(56) Documents cited
None

(58) Field of search
C1B

(71) Applicant
Stauffer Chemical Company (USA–Delaware), Westport, Connecticut 06881, United States of America

(72) Inventor
Irvin Jacob Mettler

(74) Agent and/or Address for Service
Elkington & Fife, High Holborn House, 52/54 High Holborn, London WC1V 6SH

(54) Transformation of plant cells

(57) A process for delivering a substance past the cell membrane of a cell comprising: (a) supplying an appropriate lipid mixture; (b) dissolving said lipid mixture in a solvent miscible with water; (c) forming a lipid-solvent-aqueous preparation by adding to said dissolved lipid-solvent mixture an aqueous solution of said substance; (d) intimately mixing said dissolved lipid-solvent-aqueous preparation by means non-destructive to said substance; (e) removing said solvent to form liposomes; and (g) contacting said cell with said liposomes in a suitable buffer. Plant cell protoplasts are transformed with DNA.

GB 2 140 822 A

SPECIFICATION

Liposome mediated transformation of eukaryotic cells

The invention relates to a method for encapsulating biologically active molecules, for example deoxyribonucleic acid (DNA), in lipid vesicles or liposomes and to methods of using such lipid-encapsulated DNA to transform cells, particularly plant cells.

Relevant Art

Transformation may be defined as the general process of unidirectional transfer of genetic information in which DNA originating in one cell is taken up and stably maintained by another cell. Such DNA transfers take several forms. Between bacteria, the naturally occurring transfer of information on plasmids is conjugation. Viral DNA may be relatively stably integrated into the bacterial chromsome in non-lysogenic infection only to be released at a later time in response to a derepressing stimulus.

The transformation of information in the form of naturally occurring or artificially constructed DNA from an originating source into a prokaryotic or eukaryotic cell lies at the heart of genetic engineering Such transformation may be accomplished in a number of ways. Most commonly, cells, particularly bacterial cells, are exposed for a period of time to buffered aqueous solutions of transforming DNA that contains a selectable marker. The cells are selected for the characteristic conferred by the marker. Antibiotic resistance is a commonly used selectable marker. Another transformation method entails incubating the cells to be transformed in a buffered solution containing polyethyleneglycol (PEG) at concentrations approaching 50% and a buffered aqueous solution of transforming DNA. Surviving cells are selected for the characteristics conferred by the transforming DNA.

In general, before cells can be transformed they are pretreated to render them capable of transformation. Such pretreatments allow access to the cellular membrane and subsequent transfer of the transforming DNA across the cell membrane. Before some bacterial cells can be transformed by the above briefly described techniques, bacterial cells are treated with enzymes or antibiotics that digest or interfere with formation of the bacterial cell wall. The cell membrane is thus exposed and the cells are rendered osmotically fragile. In other bacterial cells, the cells are rendered competent by transformation by exposure to calcium chloride-containing buffers.

Transformation of eukaryotic cells may be accomplished using similar techniques. Animals cells, in general, lack cell walls and are thus osmotically fragile without enzymatic or antiobiotic pretreatment. Yeast cells are generally rendered osmotically fragile by predigestion with the enzyme Zymolyse. The resulting spheroplast, which is the yeast cell lacking a cell wall, is incubated with the transforming DNA with or without PEG. Plant cells are also generally rendered osmotically fragile by enzymatic digestion of the plant cell wall prior to transformation by either method briefly described above. Such osmotically fragile plant cells are generally referred to as plant cell protoplasts.

Persons seeking to transform cells, particularly osmotically fragile cells, whether prokaryotic or eukaryotic, and particularly plant protoplasts, have encountered two major and interrelated problems in achieving transformation at high frequency. First, cells generally take up DNA through the cell membrane at very small frequencies. This phenomenon is a manifestation of a more general characteristic of the cell membrane; it is generally selectively permeable, permitting small molecules to pass through it but preventing the passage of large molecules, e.g., peptides or proteins, saccharides and polynucleotides such as DNA or ribonucleic acid (RNA). Second, in addition to the low frequency of DNA uptake, degradative enzymes, particularly nucleases, i.e, enzymes which degrade the nucleic acids DNA and RNA, present in preparations, digest the nucleic acids and disrupt the information contained therein, before it can be taken up by the cell.

A number of techniques are generally available to increase the efficiency of uptake of DNA by cells. Among these techniques is the encapsulation of aqueous solutions of the substance to be taken up by the cell in lipid vesicles, which are envelopes of lipid bilayers forming a continuous membrane that encloses an aqueous space. The lipid vesicles or liposomes are then incubated with the osmotically fragile cell under conditions that, it is believed, permit the lipid membranes of the liposome to fuse with the cell membrane of the cell to be transformed. When the lipid membrane of the liposome has fused with the cell membrane, the aqueous contents of the liposom s are, it is beli ved, r l ased into th aqueous cytosol that comprises the aqueous phas of th interior of the c ll.

Various factors aff ct the efficiency of liposome-m diat d d livery f aque us substances to th int rior of th cell. Specifically, with r sp ct to liposome-mediat d deliv ry of macromolecul s such as polynucleic acids, .g., DNA and RNA, prot ins and p lysaccharides, th ffici ncy with which th aqueous substance is encapsulated in th liposome, the stability f the substances to be encapsulated during liposome formation, and th variability in the uptake of

liposom s by different cells all affect the rate of delivery of aqueous substanc s into the cell.

An exc llent review of the factors affecting encapsulation and of the liposome field in g neral is "Liposom s: Pr paration and Characterization" by Szoka, S. and Papahadjopoulos, D., appearing as Chap r 3 of *Liposomes: From Physical Structure to Therapeutic Applications,* Elseier/North Holland Biomedical Press (1981), Knight, Editor.

Briefly summarized, the methods presently in use are as follows. The most simply formed liposomes are multilamellar vesicles (MLV). MLVs are formed by depositing a thin lipid film on a wall of a flask or tube, adding an aqueous phase and gently shaking the vessel. The MLV's thus formed have multiple lipid layers or lamellae. The major drawback of MLV is low aqueous encapsulation due to a formation of the multiple lipid layers. Bangham et al. (1965), *J. Mol. Biol.*, 13:238–252.

If MLVs are subjected to sonication under an inert atmosphere, such as nitrogen, a homogeneous population of small unilamellar vesicles (SUV) under 100 nanometers (NM) in diameter is formed. The small size of SUVs limits both the encapsulation of aqueous space per mole lipid and the size of macromolecules that can become encapsulated to generally less than about 40,000 daltons (d). Paphadjopoulos, D. and Miller, N. (1967), *Biochim. Biophys. Acta.*, 135:624–638. Adrian, G. and Huang, L. (1979) *Biochemistry*, 18:5610–5614.

Several methods have been developed to produce large unilamellar vesicles (LUV), which are lipid vesicles over 100 NM in diameter. Solvent injection, which is formation of vesicles by infusion of organic solvents containing phospholipids into a comparatively large volume of an aqueous phase, has been used but encapsulation efficiency of plasmid pBR322 DNA is only about 3 percent (%). Deamer, D.W. and Bangham, A.D. (1976), *Biochem. Biophys. Acta.* 443:629–634. Fraley, R.T. et al. (1979), P.N.A.S. 76:3348–3352. In addition, the technique is carried out at about 60°C, a temperature that is relatively high for maintaining the integrity of most biological molecules.

Another technique involving the removal, by various means, of detergents from detergent/phospholipid mixtures has been used, but encapsulation efficiencies range only from about 6% to about 12%. Enoch, H.G. and Strittmatter, P. (1979), P.N.A.S. 76:145–149. Moreover, residual detergent associated with the liposome can affect cell survival when the liposome produced by this method is employed to deliver the contents of the aqueous space to the cell interior.

Calcium induced fusion has also been used to form LUVs. Papahadjopoulos et al. (1975), *Biochem. Biophys. Acta*, 394:483–491. In this method pre-formed SUVs comprised of acidic phospholipids are treated with calcium and ethylenediaminotetraacetic acid (EDTA) to produce large closed unilamellar vesicles. The efficiency of encapsulation for particles, such as viruses and macromolecules, such as messenger RNA and DNA, is only about 10% using this technique; for smaller molecules such as sucrose about 15% encapsulaton efficiency is observed. In addition, the technique is limited by the size of the molecules that are initially encapsulated in the SUV's used in this method.

Another technique, reverse phase evaporation has been used to encapsulate substances dissolved in an aqueous phase. Depending on the ionic strength of the aqueous buffer, from 20% to about 60% of the aqueous phase may be encapsulated using this technique. Szoka, S. and Papahadjopoulos, D. (1978), *Ann. Rev. Biophys. Bioeng.*, 9:467–508; Fraley et al. (1980) *J. Bio. Chem.*, 255:10431–10438. In this method, the lipids are dissolved in organic solvents or low boiling point fluorocarbons. The aqueous material is added directly to the lipid/solvent mixture and the preparation is then sonicated to form a homogeneous emulsion. The sonication step is crucial to high encapsulation rates. The solvents are then removed by evaporation, during which step the liposomes form.

Summary of the Invention

In seeking to employ reverse phase evaporation to prepare liposomes containing DNA for transformation of plant protoplasts and other osmotically fragile cells, the inventor has discovered that the art-taught step of sonication, even for brief periods of time, substantially disrupts and degrades the DNA that is contained in the liposomes and that is ultimately transferred into the cell to be transformed. As a result, although encapsulation efficiency using the reverse phase evaporation technique is high, transformation frequency is low.

The inventor has furthermore discovered that vigorous shaking of the lipid-solvent-aqueous preparation produces liposomes with high encapsulation efficiencies without disrupting or otherwis d grading th macro-molecules, particularly DNA, contained within the aqueous spac of the liposome.

In particular, it has been discov red that liposomes, prepared by revers phase evaporation, in which the sonication step is omitted and a vortexing step is substituted th refor to emulsify the lipid-solv nt-aqu us preparation containing various plasmids, may b used to transform plant c lls with high fr qu ncy. Mor ver, it has be n discover d that plasmids can be delivered substantially intact into plant protoplasts using liposomes produced in this mann r. Specifically,

it has been discovered that liposome-encapsulated plasmid pBR327 may be transferred in high frequency into plant cell protoplasts where it is unexpectedly stably maintained and reproduced. Furthermore, it has been found that pBR327 is stably maintained and reproduces in plant cells.

The process employs an appropriate mixture of lipids for making the liposomes. Such lipids may include for example phosphatidyl serine (PS), phosphatidyl choline (PC), dipalmitidyl phosphatidyl choline, cholesterol (CH), stearylamine (SA) and dicetylphosphate (DCP). Alphatocopheral may also be added to the mixture.

The ratios of the various lipids may vary but in general the range will be within the following molar ratios:

0–10 PS : 0–9 PC : 0–5 CH : 0–1 SA : 0–1 DCP. Although it is possible to form liposomes from PS only or PC only, mixtures of lipids are more usually employed.

In general the ratio of various lipids can be varied to form liposomes having either positive, neutral or negative net charge. For example, 1 PS :4 PC : 5 CH yields liposomes having a net negative charge. 1 PC : 1 CH liposomes have a net neutral charge. 1 SA : 4 PC :5 CH liposomes have a net positive charge. Liposomes having a net negative charge are preferred.

If the various lipids are supplied in a solvent, for example chloroform, the lipids in the solvent are mixed in the desired ratio in a container; the solvent is evaporated and the lipids remain in the container. An organic solvent such as diethyl ether or diisopropyl ether, or a mixture of organic solvents, for example, diisopropyl ether and chloroform or low boiling point fluorocarbons is added to the lipid mixture. Preferably, diethyl ether is used.

An aqueous solution containing the substance to be encapsulated in the liposomes is prepared. In general, the aqueous solution will comprise the substance to be encapsulated, for example a macromolecule such as DNA, RNA, protein, polysaccharide, glycoprotein or the like, or a plasmid, in an appropriate buffer. For example, if DNA is to be encapsulated, 10 millimolar (mM) tris(hydroxymethyl)aminomethane (Tris) and 0.1–1.0 mM EDTA at pH 8.0 or 0.4 molar (M) mannitol buffer at the same pH is used. The aqueous solution is added to the lipid-solvent mixture to form a lipid-solvent-aqueous preparation.

The lipid-solvent-aqueous preparation is then vigorously agitated by means substantially non-disruptive and non-destructive to the integrity of the substance to be incorporated. Such means generally include rapid simultaneous rotation and shaking of the preparation for a period of time sufficient to allow substantial emulsificatiion of the liquid solvent and aqueous phases of the preparation. Rapid vigorous shaking alone and rapid vigorous rotation alone should also be adequate for this purpose. One device appropriate for the vigorous agitation required is a vortex mixer. Vortexing for a period of time ranging between approximately 30 seconds to 10 minutes gives adequate emulsification of the lipid/solvent/aqueous preparation. Vortexing for 3 minutes is routinely used. Sonication is not desirable since it causes degradation of the structure of many macromolecules, particularly polynucleotides such as DNA and RNA and polypeptides such as protein.

After adequate agitation to emulsify the preparation, the solvent is removed from the preparation, preferably at physiological temperatures not exceeding 37° Centrigrade (C). Although 37°C is preferred because substantial degradation of most biological molecules such as DNA, RNA polysaccharides and polypeptides does not occur at this temperature, the temperature may be increased, depending upon the resistance of the molecule to be incorporated to heat, or lowered depending on the viscosity of the lipid mixture, phase transition optimum for the lipids used and optimum temperature for liposome uptake of the cells. Optimally, the solvent is removed at negative pressure, preferably about — 12 inches of mercury (Hg) (304.mm Hg) under an inert gas carrier such as nitrogen.

The liposomes obtained as outlined above may be used directly to transform osmotically fragile cells such as plant protoplasts or the liposomes may be subjected to a purification step. Generally purification may be accomplished by analytical centrifugation, chromatagraphic or dialysis means. Liposomes may also be selected for charge by electrophoretic means. Purification may be accomplished, for example, by layering the liposome preparation on a step or linear sucrose or sucrose-osmoticum gradient in a centrifuge tube and spinning the liposome preparation at an appropriate speed and duration in a centrifuge to allow selection of the desired liposome fraction. One method used with considerable success has been to resuspend the liposomes in a sucrose solution at a concentration between about 0.4 and about 0.8 M at the bottom of a centrifuge tube. A layer of sucrose at a concentration in a range between about 0.2 to about 0.4 M and osmoticum, preferably mannitol at a concentration range between about 0.1 and 0.4M, is placed above the resuspended liposomes. Preferably, the sucrose-osmoticum solution is comprised of 0.3 M sucrose and 0.1 M mannitol. The tube is centrifuged at approximately 100,000 times gravity (x g) for approximately 40 minutes at the end of which period, liposomes that have floated to the top of the tube are collected.

Other methods for identifying liposomes having particular characteristics and isolating the identified liposomes can also be used. For example, exclusion chromatography on large pore gels and thin layer chromatography using agarose beads are well known methods for

d termining th size distribution of liposome preparati ns. Van R nswoud et al. (1980) *Biochem. Biophys. Acta.* 595:150–156.

Th lipos m preparation, whether purified r unpurified is then brought into contact with the cells to b transformed. In g neral, any osmotically fragile c ll or cell r nd r d capable of being transformed can be employed in this step but in practice the cell to be transformed will be selected for a particular purpose and complex of characteristics. Thus, for example, animal cells can be selected. Yeast cells enzymatically or antibiotically modified to form spheroplasts may be contacted if, for example, the transformed yeast cells are to be ultimately used in a fermentation, brewing or cloning process. Bacterial protoplasts or bacterial cells pretreated with calcium chloride solutions or by other methods that render the bacterial cell capable of transformation. may be used for a variety of purposes e.g., amino acid production, hormone production, cloning and the like.

Plant cell protoplasts produced by a variety of methods well known to those skilled in the art have been repeatedly transformed using liposomes containing exogenous DNA produced in the above-described manner. The conditions for transformation of plant cell protoplasts vary but in general the plant cell protoplasts will be maintained in an appropriate buffer which is designed to maintain the osmotic equilibrium of the plant cell protoplast. Such buffers are well known in the plant cell culture art. An example of one such buffer is 5 mM Tris including an osmoticum, for example mannitol, in a range of from 0.4 M to 0.8 M, and calcium chloride in a ranged from greater than about 1 mM to about 50 mM at a pH in a range from about 5.0 to about 8.0. A pH of about 6.5 is preferred for transformation.

Transformation using liposomes is facilitated by adding to the sample of buffer and the plant cell protoplasts, PEG in a molecular weight range from about 1000 to about 20,000 d and a concentration range of between about 10% to about 40% volume/volume (v/v) of total sample. PEG in a molecular weight range from about 1000 to about 6000 d is particularly suitable and a PEG molecular weight of about 4000 d in a concentration of about 20% v/v of the total sample has been found to be optimal. Using the liposome transformation system described hereinabove, it has been possible to deliver into the plant protoplasts a variety of plasmids of prokaryotic, eukaryotic, and mixed prokaryotic and eukaryotic origin. Plasmids delivered into the plant protoplasts using the methods outlined above include YeP-13LT5, *Escherichia coli* pBR322 and pBR327 and portions of the SV40 genome, however not all of these plasmids are stably maintained. Surprisingly it has been discovered that pBR327 is stably maintained in the plant cell and is stably reproduced therein.

The invention will be better appreciated by those skilled in the art from the following examples, which are intended by the interior to be exemplary only. It will be readily apparent that departure from the exact chemical concentrations, temperatures, duration of treatment, cell type and the like are expected without departing from the scope of the invention.

EXAMPLE 1

Effect of Sonication or Mechanical Vortexing on Efficiency of DNA Encapsulation by Negatively Charged Liposomes

A lipid mixture containing 1 micromolar (uM) phosphatidylserine, 4 uM phosphatidyl choline and 5 uM cholesterol was dissolved in 0.5 milliliters (ml) of ether to form a lipid-solvent mixture. A buffered aqueous solution was prepared containing 32 phosphate (32_p) labelled DNA, from plasmid BAMH/29 inserted into pBR322, at a concentration of 10 micrograms (ug) per ml in 10 mM potassium chloride, 10 mM potassium phosphate and 0.1 mM EDTA at pH 6.5. 0.15 ml of this solution was added to the lipid solvent mixture to form a lipid-solvent-aqueous preparation. The lipid solvent aqueous preparation was emulsified by sonication for a period of 5, 20 or 120 seconds in a bath-type sonicator or by mechanical vortexing in a range of 30, 120 or 600 seconds. After emulsification, the liposomes were formed by removal of the ether under partial vacuum at about − 12 inches Hg in an inert nitrogen atmosphere. The liposomes thus obtained were purified by centrifugation as follows: About 0.15 ml of the liposomes were mixed with about 0.85 ml of 0.4 M sucrose. 2.85 ml of a sucrose-osmoticum solution of 0.3 M sucrose and 0.1 M mannitol was layered above the 0.4 M sucrose liposome layer and the tube was spun at about 100,000 × g for about 40 minutes. The purified liposomes floated to the top of the tube where they were collected.

The percent DNA encapsulation was estimated by measurement of 32_p labelled DNA in a liquid scintillation counter set to read 32_p. The encapsulation efficiencies of the various treatm nts ar shown in Tabl I. The encapsulati n fficienci s using vort xing were approximat ly 65% of those achiev d by sonication for th same period of time (120 s conds). Incr asing the period of v rt xing to 600 s conds yields a DNA encapsulation rat equal to about 71% of th maximum rate obs rv d using sonication.

TABLE I

Treatment Vortexing Time	% Encapsulation of 32P-Labelled DNA		Treatment Sonication Time
30 seconds	19	31	5 seconds
120 seconds	24	38	20 seconds
600 seconds	27	37	120 seconds

EXAMPLE 2

Effect of Vortexing or Sonication on Integrity of Liposome Encapsulated DNA

Liposomes were produced as in Example 1 above. After the liposomes were formed aliquots of approximately 30 ul each of purified liposome preparation were placed in 0.25 ml micofuge tubes. 0.1 ml phenol was added and mixed thoroughly with the liposome aliquot followed by centrifugation for 30 seconds. 20 ul of the aqueous suspension remaining after the phenol extraction was removed for gel electrophoresis on a 1.4% agarose gel in 10mM TRIS, 1 mM EDTA buffer at pH 8. The samples were run for about 2.5 hours at 150 volts. The sample front was determined with blue blue tracer dye in 50% glycerol added to each sample well of the gell. The DNA fractions were visualized by treatment with ethidium bromide under ultraviolet (UV) light. Untreated DNA and DNA size standards were run on the gel at the same time. No noticeable difference was observed between vortexed samples and untreated plasmid DNA. Extensive breakage into random length fragments of the DNA of all the sonicated samples was evidenced by long DNA smears having no discernable migration peaks.

EXAMPLE 3

Liposome Mediated Delivery of Plasmid pBR327 to Corn Protoplasts

Corn protoplasts were prepared as follows. Corn suspension cells were incubated in 0.2 M mannitol, 0.08 M $CaCl_2$, 5% (w/v) Cellulysin (Cal Biochem), 2.5% Pectinase (Sigma) and 1.25% Driselase (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) at 16°C for 4 to 6 hours in flasks on a rotary incubator at 70 rpm. Corn protoplasts were collected by low speed centrifugation for 2 minutes in a clinical centrifuge. The corn protoplasts were washed twice in 0.4 M mannitol, 10 mM KCl and 10 mM 2-(N-morpholino)ethane sulfonic acid (MES)-Tris at pH 6.5 and resuspended at 1×10^6 protoplasts per ml in 0.4 M mannitol buffer. Liposomes were formed as follows: 0.81 mg PS, 3.1 mg PC, 1.9 mg CH and 25 ug alpha-tocophenol were admixed in chloroform and the chloroform was removed by vacuum evaporation. 0.5 ml of ether was added to the lipids for a final volume of about 0.5 ml. DNA from plasmid pBR327, either labelled with ^{32}P by nick translation or unlabelled was suspended at a concentration of 1mg/ml in an aqueous buffer solution containing 10 mM Tris, 1 mM EDTA at pH·8.0. 25 ul of the DNA containing aqueous buffer solution was added to the lipid-ether mixture. Water and sorbitol were added to this mixture to a final sorbitol concentration of 0.4 M with a final aqueous volume 0.15 ml forming a lipid-solvent-aqueous preparation. The lipid-solvent-aqueous preparation was vortexed for approximately 3 minutes. After vortexing the ether was evaporated in a rotary evaporator at about − 12" Hg (304.8 mmHg) under a nitrogen atmosphere at 37°C. Liposomes formed during the ether evaporation step. 0.15 ml of crude liposomes were purified by suspension in 0.85 ml of 0.4 M sucrose in a centrifuge tube at a final volume of 1 ml. The liposome-sucrose mixture was overlayed with 2.85 ml of 0.3 M sucrose and 0.1 M mannitol. The tube was spun at approximately 100,000 × g for 40 minutes at 20°C. The liposomes floated to the top forming a purified liposome preparation that was removed for incubation with the corn protoplasts.

Approximately 1 ml of the corn protoplasts in 0.4 M mannitol buffer containing 1×10^6 corn protoplasts per ml incubated with 0.1 ml of the purified liposome preparation containing approximately 0.97 mg/ml of lipid. PEG 4000 was added to this mixture to a final concentration of about 20%. The liposome protoplast PEG mixture was incubated at 25°C for approximately 15 minutes.

The liposome protoplast PEG mixture was slowly step-wise diluted with 0.4 M with mannitol buffer to final volume of approximately 10 ml. The diluted preparation was centrifuged at approximately 100 × g for 2 minutes on a low speed clinical centrifuge. The supernatant was r moved and the corn protoplasts w re resuspend d in 8 ml of 0.4 M mannitol buffer, rec ntrifuged as above and r suspended in growth media. On liter (1) of growth media c ntains the following: potassium phosphate mono-basic 170 mg, ammonium nitrate 1600 mg, postassium nitrate 1900 mg, calcium chlorid dihydrate 440 mg, magn sium sulfat 7 hydrate, 370 mg, magnanese sulfate monohydrate 16.9 mg, zinc sulfate monohydrat 10.3 mg, b ric acid 6.2 mg, potassium iodide 3.83 mg, sodium molybdate 0.25 mg, cupric sulfate 0.025 mg,

cobalt chloride 0.025 mg, nic tinic acid 5 mg, thiamine hydrochloride 10 mg, pyridoxin hydrochloride 10 mg, i-inosit l 100 mg, 2,4-dichloroph noxy acetic acid 2.0 mg, sucrose 20 g, gluc se 250 mg, mannitol 64 g, and 0.1% agar. The pH of the m dium was adjusted to 5.0. Conditioned m dia comprised f th medium described abov in which corn protoplasts are first grown for 2–4 days and then removed, is added to the growth media to a final concentration of about 20%. The corn protoplasts were grown in plates containing the media described above at 28–30°C in the dark for a period sufficient for sustained growth.

Plasmid DNA was isolated from the cells at various times during a 26 day growth period. The cells were collected by centrifugation and the supernatant growth medium was discarded. 10^4 to 10^5 cells were resuspended in 0.2 ml of extraction buffer containing 1% sarkosyl, 20 mM EDTA, 50 mM NaCl, 250 mM sucrose, 50 mM Tris, at pH 8.0 for approximately 4 hours at room temperature. The extraction buffer was washed twice with 0.5 ml of phenol saturated with 50 mM Tris, 50 mM sodium chloride, 2 ml EDTA and 1 mM beta-mercaptoethanol at pH 7.5. DNA was precipitated with ethanol and was stored overnight at − 20°C. The DNA pellet was washed and resuspended in 95% ethanol, air dried and finally redissolved in 0.1 sodium chloride-sodium citrate buffer.

Aliquots of this purified DNA preparation were used to transform *E. coli* using methods well known to those skilled in the art. After transformation and subsequent growth of the transformed *E. coli* cells, the plasmid was reisolated from the cultured *E. coli* cells. The reisolated plasmid DNA was digested with restriction enzymes and electrophorised alongside the plasmid DNA previously isolated from the plant cells for comparison purposes.

DNA isolated from the plant cells was resolved by electrophoresis on a 1.4% agarose gel. The DNA was blotted onto nitro cellulose using the Southern blot method and hybridized to a ^{32}P-labelled DNA probe of pBR327. The resulting filter is autoradiographed on Kodak X-omat film.

No difference was found between the pBR327 DNA isolated from the plant protoplasts or *E. coli* and the pBR327 standards. Significantly, the pBR327 DNA bands increased in intensity with the increase in period of the corn protoplasts culture, indicating that pBR327 plasmid reproduced within the corn cell cultures.

CLAIMS

1. A process for delivering a substance past the cell membrane of a cell comprising:
(a) supplying an appropriate lipid mixture;
(b) dissolving said lipid mixture in a solvent miscible with water;
(c) forming a lipid-solvent-aqueous preparation by adding to said dissolved lipid-solvent mixture an aqueous solution of said substance;
(d) intimately mixing said dissolved lipid-solvent-aqueous preparation by means non-destructive to said substance;
(e) removing said solvent to form lipsomes; and;
(g) contacting said cell with said liposomes in a suitable buffer.

2. The process defined in Claim 1 wherein said solvent is a polar organic solvent evaporable in conditions non-destructive to said substance.

3. The process defined in Claim 2 wherein said solvent is ethyl ether.

4. The process of Claim 1 wherein said removing step is by evaporation at about 37°C at − 12″ (− 304.9% mm) Hg under an appropriate gas carrier.

5. The process of Claim 1 wherein said intimately mixing step is mixing other than sonication.

6. The process of Claim 5 wherein said mixing step is vortexing.

7. The process of Claim 1 wherein said appropriate lipid mixture is selected from a group of lipids consisting of phosphatidyl serine, phosphatidyl choline, dipalmityl phosphatidyl chloline, cholesterol, stearylamine and dicetylphosphate.

8. The process of Claim 7 wherein said lipids further include alpha-tocophoral.

9. The process of Claim 7 wherein the molar ratios of said lipids are in a range of 0–10:0–9:0–5:0–1 for phosphatidyl serine, phosphatidyl choline:cholesterol:stearylamine:diacetylphosphate, respectively.

10. The process of Claim 9 wherein said molar ratio is 1:4:5 for phosphatidyl serine, phosphatidyl choline and cholesterol, respectively.

11. The process of Claim 9 wherein said molar ratio is 1:1 for phosphatidyl choline and cholesterol, respectively.

12. The process of Claim 9 wher in said molar ratio is 1:4:5 for stearylamin , ph sphatidyl choline and cholesterol, respectively.

13. Th pr cess of Claim 1 wher in said liposomes hav a n t n gative charg .

14. The process of Claim 1 wherein said liposomes hav a n t n utral charge.

15. Th process of Claim 1 wh rein said liposomes have a net positiv charge.

16. The process of Claim 1 wherein said substanc to be d livered is a saccharide.

17. The proc ss of Claim 1 wh r in said substanc to be delivered is a p ptide.

18. The process of Claim 1 wherein said said substance to be delivered is nucleic acid.
19. The process of Claim 1 wherein said substance to be deliv r d is a macromol cule.
20. The process of Claim 19 wherein said macromolecul is a polysaccharid .
21. The process of Claim 19 wherein said macromolecule is a polypeptid .
22. The process of Claim 19 wherein said macromolecule is a polynucleic acid.
23. The process of Claim 22 wherein said polynucleic acid is RNA.
24. The process of Claim 22 wherein said polynucleic acid is DNA.
25. The process of Claim 22 wherein said polynucleic acid is a plasmid.
26. The process of Claim 25 wherein said plasmid is Yep13 LT5.
27. The process of Claim 25 wherein said plasmid is pBR322.
28. The process of Claim 25 wherein said plasmid is pBR327.
29. The process of Claim 28 wherein said plasmid replicates in said cell.
30. The process of Claim 22 wherein said nucleic acid is a vector.
31. The process of Claim 22 wherein said vector is yEP13LT5.
32. The process of Claim 22 wherein said vector is pBR322.
33. The process of Claim 22 wherein said vector is pBR327.
34. The process of Claim 33 wherein said vector replicates in said cell to be transformed.
35. The process of Claim 1 wherein said cells to be contacted are prokaryotic cells.
36. The process of Claim 35 wherein said cells to be contacted are osmotically fragile or competant prokaryotic cells.
37. The process of Claim 1 wherein said cells to be contacted are osmotically fragile eukaryotic cells.
38. The process of Claim 37 wherein said cells to be contacted are plant protoplasts.
39. The process of Claim 37 wherein said cells to be contacted are yeast spheroplasts.
40. The process of Claim 37 wherein said cells to be contacted are animal cells.
41. The process of Claim 1 wherein said contacting step further comprises the steps of:
(i) suspending said cells to be contacted in an appropriate buffer;
(ii) adding said liposomes to said suspended cells;
(iii) incubating said suspended cells and liposomes together at an appropriate temperature;
(iv) adding polyethyleneglycol to said incubating suspended cells and liposomes;
(v) incubating said liposomes and suspended cells for a period of time sufficient to allow fusion of said liposomes and cells.
42. The process of Claim 41 wherein said cells to be contacted are prokaryotic cells.
43. The process of Claim 41 wherein said cells to be contacted are osmotically fragile or competant prokaryotic cells.
44. The process of Claim 41 wherein said cells to be contacted are osmotically fragile eukaryotic cells.
45. The process of Claim 44 wherein said cells to be contacted are plant protoplasts.
46. The process of Claim 44 wherein said cells to be contacted are yeast spheroplasts.
47. The process of Claim 44 wherein said cells to be contacted are animal cells.
48. The process of Claim 41 wherein said incubation step is for about 5 minutes to 60 minutes at about 16 to 28°C.
49. The process of Claim 41 wherein said incubation step is for about 20 minutes at about 25°C.
59. The process of Claim 41 wherein said polyethyleneglycol is in a molecular weight range from about 1000 to about 20,000 daltons and the concentration thereof is from about 10% to about 40% of the total solution.
51. The process of Claim 41 wherein said polyethyleneglycol has a molecular weight range from about 1000 to about 6000 daltons and the final concentration thereof is about 20% of the total solution.
52. The process of Claim 41 wherein said polyethyleneglycol has a molecular weight of about 4000.
53. The process of Claim 41 wherein said appropriate buffer comprises calcium chloride in a concentration range from about greater than 1mM to about 50 mM, osmoticum in a concentration range from about 0.4 M to aobut 0.8 molar, Tris HCl in a concentration about 5 mM, and a pH range from about 5.0 to 8.0.
54. The process of Claim 41 wherein said appropriate buffer comprises about 10 mM calcium chloride, about 0.4 molar mannitol, about 5 mM Tris HCl and a pH of about 6.5.
55. The process defined in Claim 1 wherein said s lv nt is polar organic solv nt evaporable in conditions non-destructive to said substance.
56. The proc ss d fin d in Claim 55 wherein said purifying st p furth r c mprises th st ps of:
(i) resuspending said liposomes in a volume of a sucrose solution;
(ii) overlaying said sucrose solution with a volume of a sucrose-osmoticum solution;
(iii) centrifuging said resuspended lip somes for a period sufficient to separate substances n t

contained within said liposomes therefrom;

(iv) collecting said liposomes after they have floated to the surface of said sucrose-osmoticum; and

(v) resuspending said liposomes in a buffer.

57. The process of Claim 56 wherein said sucrose solution is in a range from about 0.4 molar to about 0.8 molar and said sucrose osmoticum solution is comprised of sucrose in a range from about 0.2 to about 0.4 molar and mannitol in a range from about 0.1 to about 0.4 molar.

58. The process of Claim 56 wherein said is sucrose is in an amount of 0.4 molar and said sucrose-mannitol solution is 0.3 molar sucrose and 0.1 molar mannitol.

59. The process of Claim 56 wherein said sucrose and sucrose-mannitol solution are in about a 1:3 volume to volume ration.

60. The process of Claim 56 wherein said purified liposomes comprise about 2 micromoles of liposomal lipid per 10^6 of said cells to be contacted.

61. The process of transformation plant cells comprising:

(a) forming plant cell protoplasts;

(b) transforming said plant cell protoplasts with DNA.

62. The process of Claim 61 wherein said DNA is a plasmid.

63. The process of Claim 62 wherein said plasmid is pBR327.

64. The process of Claim 61 wherein said DNA is liposome encapsulated.

65. The process of Claim 64 wherein said DNA is encapsulated by the steps of

(a) supplying an appropriate lipid mixture;

(b) dissolving said lipid mixture in a solvent miscible with water;

(c) forming a lipid solvent aqueous preparation by adding to said dissolved lipid-solvent mixture said DNA in an aqueous solution;

(d) intimately mixing said dissolved lipid-solvent-aqueous preparation by means non-destrictive to said DNA; and

(e) removing said solvent to form liposomes.

66. The process of Claim 65 wherein said DNA is a plasmid.

67. The process of Claim 66 wherein said plasmid is pBR327.

68. Plant cells transformed by the process of Claim 61.

69. The plant cells of Claim 61 wherein said DNA is a plasmid.

70. The plant cells of Claim 69 wherin said plasmid is pBR327.

71. The plant cells of Claim 68 wherein said DNA is liposome encapsulated.

72. The plant cells of Claim 64 wherein said DNA is encapsulated by the steps of:

(a) supplying an appropriate lipid mixture;

(b) dissolving said lipid mixture in a solvent miscible with water;

(c) forming a lipid solvent aqueous preparation by adding to said dissolved lipid-solvent mixture said DNA in an aqueous solution;

(d) intimately mixing said dissolved lipid-solvent-aqueous preparation by means non-destructive to said DNA; and

(e) removing said solvent to form liposomes.

73. The plant cells of Claim 72 wherein said DNA is a plasmid.

74. The plant cells of Claim 73 wherein said plasmid is pBR327.

Printed in the United Kingdom for Her Majesty's Stationery Office, Dd 8818935, 1984, 4235.
Published at The Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.